

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Micronuclei frequency in peripheral blood lymphocytes of healthy subjects living in Turin (North-Italy): contribution of body mass index, age and sex.

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1725067> since 2020-01-24T13:53:26Z

Published version:

DOI:10.1080/03014460.2020.1714728

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



Micronuclei frequency in peripheral blood lymphocytes of healthy subjects living in Turin (North-Italy): contribution of body mass index, age and sex.

Journal:	<i>Annals of Human Biology</i>
Manuscript ID	TAHB-2019-0124.R1
Manuscript Type:	Research Paper
Keywords:	environmental pollution, Italian population, genomic damage, genotoxicology

SCHOLARONE™
Manuscripts

Response to Referee 1:

Referee 1: "This study describes more general frequency of CBMN assay biomarkers and could be regarded as the study giving baseline values of MN assay parameters. Saying that this is connected with the air pollution because Turin is regarded as one of the most polluted cities in Italy is a bit overestimated. To connect MN frequency with the biomarkers of exposure to air pollution one should measure biomarkers of exposure or at least present certain data regarding air pollution in the time frame of the blood sampling to access the exact levels of air pollutants during the sapling of population. Without that, this paper is simply biomonitoring study undertaken to access baseline frequency of MN assay parameters in a healthy population of the city of Turin. In line with that authors should change the title and the aim of the study. Trough discussion section one could hypothesis that the higher MN frequency could be due to air pollution but nothing more".

Authors: In agreement with the referee, we changed title and aim of the study. Introduction and Discussion sessions were modified, trying to give more emphasis to the population aspects. Moreover, as suggested by the referee 2, the introduction session was improved with some concepts about the evolutionary meaning of MNI.

Referee 1: "Authors could even give some data on the air pollution parameters form their city in the year of blood sampling but without that this seems a bit overestimated to say that the higher frequency is due to air pollution since it could be from several other lifestyle factors, dietary preferences, polluted water etc".

Authors: In agreement with the referee, we provide table (Table 5) with data about air pollution in Turin in 2017, year of sampling

Referee 1: "Page 6, line 33 – use \times instead of X"

Authors: In agreement with the referee, we modified the text as suggested.

Referee 1: "Page 6, line 38 - A total of 1,000 lymphocytes per donor per concentration – what do you mean by per concentration? It should be per donor only?"

Authors: We apologize, it was a mistake. In the new version "per concentration" was deleted.

Referee 1: "Please use only 2 decimal places throughout the manuscript"

Authors: In agreement with the referee, we reduced the number of decimal to 2.

Referee 1: "Page 7, line 43 – please use kg instead of Kg"

Authors: In agreement with the referee, we substituted Kg with kg.

Referee 1: "Page 8, line 5 – change subtitle to CBMN parameters frequency since all the cytome assay parameters were scored and not only MNI"

Authors: we modified subtitle, as suggested.

Resposne to Referee 2:

Referee 2: ".....An important aspect of these objectives is to learn if the biomarkers are "elevated". The statistical methods do not describe any means to test whether the biomarkers are elevated". Moreover: ".....Overall, this paper does not determine if the levels are elevated as there is no formal comparison of levels across populations. Either the objectives must be changed or the comparison added to the paper. I believe the comparison would increase the value of the paper substantially". Moreover: "Page 9, Lines 14-18. In comparing the Turin levels to those from other Italian towns, It is appropriate to test the difference before concluding that the Turin values are higher. As the objective is to detect elevated levels, this comparison should be part of the results".

Authors: We agree with referee 2. It would have been very interesting to be able to perform a comparative statistics with published results for other Italian cities. However, as also mentioned in the new version of discussion session, it was not possible because "...although in some cases numerical differences seem to be relevant, it should be emphasized that these comparisons between cities are not supported by a statistical test, due to the lack of available published data about the frequency of MNI relating to each subject participating to various published studies".

Based on these considerations, we change title and, partially, the objectives of the work, as also suggested by the referee 1. Moreover, Introduction and Discussion sessions were modified, giving more

emphasis to the population aspects.

Referee 2: "Page 7. Line 19: No need to report mean values of age and other variables to the thousands (32.712 = 37.7), nor should they be reported to some many decimals in the tables. The rule of significant digits applies here".

Authors: In agreement with the referee, we reduced the number of decimal to 2, as also suggested by referee 1.

Referee 2: "The discussion does not discuss the implications of the levels of the biomarkers for health or evolution. What is the significance of the findings?"

Authors: The possible implications for human health of increased levels of MNi was discussed in both Introduction and discussion sessions. In particular, in the introduction session, we stated: "....increased levels of MNi in PBL have been associated prospectively with increased risk of cancer, cardiovascular and neurodegenerative diseases.....". Moreover, in the discussion session we reiterated that "Prolonged human exposure to high levels of urban pollution was associated with increased risk of cancer, respiratory and cardiovascular diseases, as well as a relationship was found between high levels of MNi in peripheral blood lymphocytes and increase of cancer risk.....".

Relatively to the evolutionary implications of increased levels of MNi, in the introduction session, we added the following paragraph:

"....MNi do not represent just the products of biological errors, but, recently, other biological effects were associated to their formation. In particular, exposing fragmented DNA to the cytoplasm, MNi triggers the activation of immunity system related genes, suggesting that the presence of MNi can be sensed by the same immunity system (Gekara, 2017). Moreover, MNi also represent a mechanism of elimination of genetic material, such as amplified genes, and contribute to nuclear dynamics and genome chaos (Heng, 2019; Ye CJ 2018). This last represents a process of rapid genome re-organization that results in the formation of chaotic genomes, some of which could be selected to establish stable genomes (for a review see Ye et al., 2019)".

We decided to insert this paragraph in the introduction session instead of discussion session, as indicated by the referee 2, because it seemed us to fit better into the introduction rather than the discussion session, and because we decided to limit the discussion mainly to the obtained results. However, if referee 2 requires it, we can consider to move this paragraph in the discussion session.

Referee 2: "Page 11, Line 19. What is the "natural frequency" and how is it different from the frequency?"

Authors: we changed the term "natural" with the more appropriate term "baseline".

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

**Micronuclei frequency in peripheral blood lymphocytes of healthy subjects living in
Turin (North-Italy): contribution of body mass index, age and sex.**

For Peer Review Only

Abstract

Background: Increased micronuclei (MNi) frequencies in human lymphocytes are an indicator of spontaneous chromosome instability and could be influenced by different exogenous and endogenous factors. Moreover, the increased exposure to environmental pollutants has led to the awareness of the necessity for constant monitoring of human populations, especially those living in urban areas.

Aim: We used the MNi assay to evaluate the baseline genome damage in 150 subjects belonging to the general population of Turin (North-Western Italy).

Subjects and Methods: MNi, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored in 1,000 lymphocytes per subject. A possible effect of body mass index, age and sex on the amount of the level of genomic damage was evaluated.

Results: The MNi, NPBs and NBUDs average frequencies ($\% \pm \text{S.D.}$) were 7.19 ± 2.51 , 1.65 ± 1.54 and 2.07 ± 1.76 , respectively. A significant correlation was found between MNi, NPBs, NBUDs frequencies and age and body mass index.

Conclusion: Baseline frequency of MNi was established in a sample of Turin population that, like all urban populations, is exposed to high levels of environmental pollutants. We hope that the results of this study can be used as a stimulus for future biomonitoring programs in other Italian and globally distributed cities.

Key words: environmental pollution; Italian population; genomic damage, genotoxicology

Introduction

Human populations are increasingly exposed to a variety of environmental pollutants that may increase the genomic instability and may affect the baseline values of cytogenetic markers. Populations living in urban areas resulted particularly exposed, principally due to the increase of pollutants generated by industrial activities and motor vehicle exhausts. Moreover, the rise of population and a consumer-oriented economic approach have contributed to increasing atmospheric contamination with harmful substances. The consequence of this negative synergy is that about 90% of European citizens residing in urban areas are exposed to air pollution exceeding EU limit values (EC 2008) and WHO guideline levels (Ceretti et al. 2014; WHO 2006, 2007; EAA 2012).

One of the objectives for public health is to construct systems that allow the biomonitoring of the health status of people living in urban areas and, in general, of the biome. In this sense, biomarkers such as DNA and/or protein adducts, and cytogenetic alterations (e.g., micronuclei, chromosomal aberrations), may help in evaluation of genomic damage and facilitate health risk assessments.

Cytokinesis-block micronucleus (CBMN) cytome is one of the most used assay for the assessment of the level of genomic damage in various human populations (Villarini et al., 2018; Gajski et al., 2018; Nefic and Handzic, 2013; Landi et al., 2000). Evaluation of spontaneous MNi frequency can also be useful in order to determine that background level of genomic damage that represents an important comparison value when spontaneous MNi frequency for exposed individuals is not known prior to exposure (Nefic and Handzic, 2013).

The aim of this study was to evaluate the MNi frequency, as a biomarker of DNA damage, in peripheral blood lymphocytes (PBL) of non-occupationally exposed subjects living in Turin (North-West Italy), a city characterized by high levels of air pollution and for which no data are present in literature about background frequency of micronuclei.

These last represent small extranuclear bodies which have not been to be included in the daughter nuclei at telophase. They may arise from chromosome breakage or if a whole chromosome lags behind at anaphase and fails to be incorporated in the new nuclei (Fenech et al. 2011). Baseline MNi frequencies in cultured human lymphocytes represent accumulated genetic damage appearing during the lifespan of the circulating lymphocytes. However, increased levels of MNi in PBL have been associated prospectively with increased risk of cancer, cardiovascular and neurodegenerative diseases (Bonassi et al. 2011).

MNi do not represent just the products of biological errors, but, recently, other biological effects were associated to their formation. In particular, exposing fragmented DNA to the cytoplasm, MNi triggers the activation of immunity system related genes, suggesting that the presence of MNi can be sensed by the same immunity system (Gekara, 2017).

Moreover, MNi also represent a mechanism of elimination of genetic material, such as amplified genes, and contribute to nuclear dynamics and genome chaos (Heng, 2019; Ye CJ 2018). This last represents a process of rapid genome re-organization that results in the formation of chaotic genomes, some of which could be selected to establish stable genomes.

Chromosomal instability was also measured by scoring nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). NPBs represent dicentric chromosomes or the result of a defective separation of sister chromatids at anaphase, whereas NBUDs represent the process of elimination of amplified DNA and/or excess chromosomes from aneuploid cells (Fenech et al. 2011).

Finally, it is known that diet, age and sex may influence genome integrity (Fenech et al., 2003; Nefic and Handzic, 2013). Based on these assumptions, we also decided to evaluate the incidence of body mass index, age and sex on the frequency of micronuclei.

Materials and Methods

Population sample

The study population included 150 blood donors randomly sampled in Turin (Italy), during winter 2017. Individuals selected for this study were subjects without any known exposure to specific xenobiotics, except for those of the routine household and traffic. All subjects were living in the city, away from landfills, and their houses were equipped with modern heating systems. However, in the inner suburbs of the city there is an important automotive factory and numerous other smaller industrial installations that significantly contribute to the air pollution of the urban area.

In order to assess the possible influence of the age and body mass index (BMI) on the level of DNA damage, subjects were split into three age groups (21-30, 31-40, 41-55 age groups) and two BMI groups: normal weight, with BMI ranging between 18.5 and 24.99, and overweight with BMI >25.

It is well known that cigarette smoke, alcohol consumption, drugs and X-rays could alter level of genomic damage. For this reason, we excluded from the sample smokers and individuals who reported alcohol consumption, treatment for acute infections and/or chronic non-infectious diseases, history of cancer and exposure to diagnostic X-rays, for at least one year prior the analysis.

Subjects received detailed information about aims and experimental procedures of the study and gave their informed consent. Volunteers, in healthy conditions when sampling was conducted, were selected and anonymously identified by a numeric code.

The study was approved by the University of Turin ethics committee and was performed in agreement with the ethical standards laid down in the 2013 Declaration of Helsinki.

Blood Sample Collection and Cell Cultures

Blood samples were obtained by venipuncture (5-10 mL) and collected in heparinised tubes.

All blood samples were coded, stored at 4°C, and processed within 2 hours after collection.

Heparinised venous blood (0.3 mL) were cultured in 25 cm² flasks containing 6 mL of RPMI-1640 medium, 2 mL of foetal calf serum (FCS), 200 µL of the mitogenic agent

Phytohemagglutinin-L (2.3% v/v), and 100 µL of antibiotics solution (100 IU/mL penicillin, and 100 µg/mL streptomycin), for a total of 8.6 mL for each lymphocyte culture. The cultures were successively incubated at 37°C and under 5% of CO₂ in the air in a humidified atmosphere.

After 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 µg/mL to block cytokinesis.

After 72 hrs (for MNi assay) of incubation at 37°C, the cells were collected by centrifugation and treated for 10 min with a pre-warmed mild hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a fresh mixture of methanol/acetic acid (3:1 v/v). The treatment with the fixative was repeated three times.

Finally, the supernatant was discarded and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect MNi by conventional staining with 5% Giemsa (pH 6.8) prepared in Sørensen buffer.

Microscope analysis was performed at 400x magnification on a light microscope (Dialux 20, Leitz, Germany). MNi, nucleoplasmic bridges (NPB) and nuclear buds (NBUD) were scored in 1,000 binucleated lymphocytes with well-preserved cytoplasm per subject. A total of 1,000 lymphocytes per donor were scored to evaluate the percentage of cells with 1-4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated, according to the following formula: $[1 \times N1] + [2 \times N2] + [3 \times (N3 + N4)]/N$, where N1-N4 represents the number of cells with 1-4 nuclei, respectively, and N is the total number of cells scored.

Statistical analysis

Statistical analyses were conducted using the SPSS software statistical package programme (version 24.0, Inc., Chicago, Illinois, USA). Differences between sexes, age and BMI groups were evaluated by ANOVA, Wilcoxon Mann-Whitney *U* test and regression analysis. All *P*-values were two tailed and the level of statistical significance was set at *P*<0.05 for all tests.

Results

General characteristics of the studied population

The demographic and genotypic features of the investigated urban population are reported in Table 1. Sixty-six subjects were males, with mean age of 32.71±8.94, Body Height (BH) of 1.72±0.08, Body Mass (BM) of 69.21±10.25 and BMI of 23.41±2.86, whereas 84 were females, with mean age of 29.00±8.72, BH of 1.70±0.09, BM of 66.61±11.28 and BMI of 22.95±2.92). The age of the individuals included in the total sample ranged from 21 to 55 years, with a mean (±S.D.) of 30.63 (±8.979).

The total sample was split into three groups according to the age of the subjects: 21-30 (*n* = 99, with mean age of 25.12±2.74, BH of 1.69±0.08, BM of 64.90±10.30 and BMI of

22.72±2.90); 31-40 (n = 28, with mean age of 35.79±2.54, BH of 1.73±0.08, BM of 73.04±10.60 and BMI of 24.32±2.93); and 41-55 (n = 23, with mean age of 48.09±3.97, BH of 1.76±0.07, BM of 73.61±9.14 and BMI of 23.61±2.43).

Finally, total mean BH, BM and BMI were 1.71±0.08 m, 67.75±10.88 kg and 23.13±2.91 kg/m², respectively. The number of normal weight subjects was 111, with mean age 29.32±8.33, mean BM of 64.34±9.85 kg, mean BH of 1.71±0.08 m and mean BMI 21.83±2.05 kg/m². The number of overweight subjects was 39, with a mean age of 34.39±9.79, mean BH of 1.70±0.08 m, mean BM of 77.46±7.28 kg and mean BMI of 26.85±1.36 kg/m².

CBMN parameters frequency

Detailed results of the MNi analyses are summarized in Tables 2 and 3. In figure 1 some examples of observed bi-nucleated cells with MNi, NPBs and NBUDs were showed.

A total of 150,000 cells were available for the MNi, NPBs, NBUDs and CBPI analyses, respectively.

The observed MNi, NPBs and NBUDs values (‰) were 7.19±2.51, 1.65±1.54 and 2.07±1.76, respectively, while the mean CBPI value was 1.71±0.08. The regression and non-parametric analyses revealed significant differences in the level of genomic damage between normal weight and overweight subjects, with this last group showing the higher amount of genomic damage. Similarly, significant differences were found between age groups, with the younger group (21-30) showing significant lower frequencies of all genomic markers with respect to the other two age groups (Tables 2-4). Finally, no differences were found between sexes in the frequency of MNi, NPBs and NBUDs (Tables 2-4).

Discussion

In their daily activity, people are exposed to a wide variety of environmental pollutants, some of which with known genotoxic properties. In particular, urban air is a complex mixture of many different chemical species (Feretti et al. 2014), most of them with carcinogenic properties (WHO 2013). In this scenario, human biomonitoring programs provide valuable information about environmental exposure and can help to identify potential health risks. Indeed,

The aim of the present study was to evaluate the MNi frequency, as a biomarker of DNA damage, in peripheral blood lymphocytes of non-occupationally exposed subjects living in Turin (North-West Italy).

The frequency of micronucleated cells observed in our sample (7.17%) falls in the range of 3-12% established for non-exposed subjects by the Human Micronucleus (HUMN) international collaborative study, that also established a mean value of 6.5% (Nefic and Handzic, 2013; Bonassi et al., 2001). Comparing our data with those published for other Italian populations, we observed for Turin a frequency of MNi higher than that reported by Villarini et al. (2018) and Landi et al. (2000) for other Italian towns (Brescia, Pisa, Perugia and Lecce), but lower with respect to Rome (Leopardi et al. 2003) and Bologna (Maffei et al. 2014). Similarly, Turin also shows one of the highest MNi frequency with respect to other European regions, such as Croatia (5.06±3.11, Gajski et al. 2018), Denmark, (2.80±1.90, Look et al. 2014), Crete (2.85±2.15, Look et al. 2014), UK (2.77±2.54, Look et al. 2014), Spain (1.79±1.50, Look et al. 2014) and Norway (1.90±1.46, Look et al. 2014). However, although in some cases numerical differences seem to be relevant, it should be emphasized that these comparisons between cities are not supported by a statistical test, due

to the lack of available published data about the frequency of MNi relating to each subject participating to various published studies.

A possible explanation of this relative high incidence of MNi observed in Turin, could be that, because of the heavy industrial activity and the significant increase in the number of cars in the last decades, in Turin a high atmospheric contamination by particulate material and nitrogen oxides has been registered (ARPA 2019, Table 5). Moreover, Turin is a city located in the Po river valley, an area where air exchanges are limited by the surrounding mountains, winds are weak, and air pollutants can accumulate easily. As consequence, Turin is one of the most polluted European cities in terms of particulate matters and ozone (WHO 2013), whose mutagenic potential has been suggested in a number of studies (Alves et al., 2016).

Prolonged human exposure to high levels of urban pollution was associated with increased risk of cancer, respiratory and cardiovascular diseases, as well as a relationship was found between high levels of MNi in peripheral blood lymphocytes and increase of cancer risk (Bonassi et al. 2011).

However, the observed differences in the level of cytogenetic damage among different cities could be also related to differences in the local environmental conditions, as well as there are many different unpredictable agents that can cause an increase in the background frequency of genetic end-points (Rossner et al. 2013). Indeed, spontaneous MNi can also be induced by a variety of exogenous and endogenous factors, such as genotoxicant compounds in food (Santovito et al. 2012), age of individuals (Cho et al., 2017), genetic susceptibility and individual damage repair capacity (Santovito et al. 2017). In particular, among these factors, sex, age and BMI seem to play a crucial role (Gajski et al., 2018; Cho et al., 2017; Domnez-Altuntas et al., 2014) and were evaluated in the present study.

As for the role of sex, no significant differences we observed between females and males in the frequencies of MNi, NPBs and NBUDs (Tables 2 and 3). This result contrasts with that recently observed by Gajski et al. (2018) with the same cytogenetic biomarker. A convincing explanation for a possible gender difference in genomic damage levels is still missing. Different genetic history of populations, as well as polymorphisms in damage repair genes may, at least in part, explain these conflicting data.

It is known that age is an important factor to be taken into consideration when designing human population studies. According to a number of studies on age-related genomic damage incidence in control populations (Cho et al., 2017; Bonassi et al., 2001), the results obtained in this study suggest an influence of the age on MNi rates. Indeed, we observed a significant increase of the frequency of MNi, but not of NPBs and NBUDs, in the older groups compared to the younger ones (Tables 2 and 3). These data could be interpreted as a signal of a potential increase of defects in DNA repair processes with age, resulting in a progressive accumulation of genomic damage and consequent increase risk of cancer (Garcia-Sagredo 2008).

Finally, in our sample a correlation was found between BMI and genomic damage measured in terms of MNi, NPBs and NBUDs (Tables 2-4), with overweight subjects showing significant higher level of these cytogenetic markers with respect to normal weighted individuals. This result confirms data obtained by Domnez-Altuntas et coll (2014), who observed higher frequencies of MNi, NPBs and NBUDs in obese subjects with respect to normal-weight and over-weight subjects. This increase in the frequencies of MNi, NPBs and NBUDs in over-weight with respect to normal-weight subjects could be related to an increase of oxidative stress levels, and consequently to an increase of genomic damage, that some authors observed in obese and over-weight subjects. For example, Elwakkad et al. (2011), observed higher level of serum 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative stress marker, in severely obese adolescents and, in general, a positive correlation between BMI and

serum 8-OHdG concentration. Similarly, Scarpato et al (2011) reported significantly increased MNi frequencies in over-weight and obese children with respect to normal-weight children.

Conclusion

With this paper, we aimed to establish the **baseline** frequency of MNi in a sample of the urban population of Turin that, like all urban populations, is exposed to high levels of environmental pollutants. This type of data assumes a more important connotation if we consider the fact that, in the present study, as well as in other studied conducted on control populations, we analysed subjects not exposed for professional reasons to xenobiotics, but that are simply living in a city, like Turin, with many problems related to urban pollution (Raaschou-Nielsen et al. 2013; Santovito et al. 2016). In this sense, we hope that the results of this study can be used as a stimulus for future biomonitoring programs in other Italian and globally distributed cities. Finally, it should be emphasize that the results of the present work cannot be generalized for all “Caucasians” because this group is heterogeneous, with differences in the distribution of genetic polymorphisms and in life styles among individuals.

Acknowledgements

The authors would like to thank all of the volunteers who participated in this study. This study was financed by University of Turin with local 2015-2018 grants.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Agenzia Regionale Protezione Ambientale (ARPA) 2019. Piano Regionale per la qualità dell'aria. Web site:
https://www.regione.piemonte.it/web/sites/default/files/media/documenti/2019-04/prqa_allegato1_def.pdf. Accessed on 04-19-2019.
2. Alves DK, Kummrow F, Cardoso AA, Morales DA, Umbuzeiro GA 2016. Mutagenicity profile of atmospheric particulate matter in a small urban center subjected to airborne emission from vehicle traffic and sugar cane burning. *Environ Mol Mutagen*. 57(1):41-50.
3. Bonassi S, Fenech M, Lando C, Lin YP, Ceppi M, Chang WP, Holland N, Kirsch-Volders M, Zeige et al. 2001. HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ Mol Mutagen*. 37:31-45
4. Bonassi S, El-Zein R, Bolognesi C, Fenech M 2011. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis*. 26: 93-100. DOI: 10.1093/mutage/geq075
5. Ceretti E, Feretti D, Viola GC, Zerbini I, Limina RM, Zani C, Capelli M, Lamera R, Donato F, Gelatti U 2014. DNA damage in buccal mucosa cells of pre-school children exposed to high levels of urban air pollutants. *PLoS One*. 9: e96524. DOI: 10.1371/journal.pone.0096524
6. Cho NY, Kim KW, Kim KK 2017. Genomic health status assessed by a cytokinesis-block micronucleus cytome assay in a healthy middle-aged Korean population. *Mutat Res/Genetic Toxicol Environ Mutagen* 814:7-13
7. Domnez-Altuntas, H, Sahin F, Bayram F, Bitgen N, Mert M, Guclu K, Hamurcu Z, Arıbas S, Gundogan K, Diri H 2014. Evaluation of chromosomal damage, cytostasis,

- cytotoxicity, oxidative DNA damage and their association with body-mass index in obese subjects. *Mutat Res Genet Toxicol Environ Mutagen*. 771: 30-36.
8. EAA (European Environment Agency) 2012. Air Quality in Europe 2011. European Environment Agency, Technical report No 4/2012. ISSN 1725-9177
9. Elwakkad A, Hassan NM, Sibaii H, El-Zayat S, Sherif L, Hameed ERA, Shaheed AA 2011. Relationship between obesity and 8-hydroxy-2-deoxy guanosine as an oxidative marker in obese adolescents of Giza. *J Med Sci*. 11: 231-235.
10. European Commission (EC) 2008. Air Quality Directive 2008/50/EC. Web site: <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008L0050&from=EN>. Accessed on 10-01-2018.
11. Fenech M, Bonassi S, Turner J, Lando C, Ceppi M, Chang WP, Holland N, Kirsch-Volders M, Zeiger E et al. 2003. Intra- and inter-laboratory variation in the scoring of micronuclei and nucleoplasmic bridges in binucleated human lymphocytes. Results of an international slide-scoring exercise by the HUMN project. *Mutat Res* 534: 45-64.
12. Fenech M, Kirsch-Volders M, Natarajan AT, Surrallés J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P 2011. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*. 26: 125-132.
13. Feretti D, Ceretti E, De Donno A, Moretti M, Carducci A, Bonetti S, Covolo L, Bagordo F, Schillirò T, Limina RM, et al. 2014. Monitoring air pollution effects on children for supporting public health policy: the protocol of the prospective cohort MAPEC study. *BMJ Open* 4: e006096.
14. Gajski G, Gerića M, Oreščaninb V, Garaj-Vrhovaca V 2018. Cytokinesis-block micronucleus cytome assay parameters in peripheral blood lymphocytes of the general

- population: Contribution of age, sex, seasonal variations and lifestyle factors. *Ecotoxicol Environ Saf.* 148: 561-570.
15. Garcia-Sagredo JM 2008. Fifty years of cytogenetics: a parallel view of the evolution of cytogenetics and genotoxicology. *Biochim Biophys Acta.* 1779: 363-375.
16. Gekara NO. 2017. DNA damage-induced immune response: Micronuclei provide key platform. *J Cell Biol* 216:2999-3001.
17. Heng HH. 2019. *Genome Chaos: Rethinking Genetics, Evolution, and Molecular Medicine*; Academic Press Elsevier:Cambridge, MA, USA, 2019; ISBN 978-012-8136-35-5.
18. Landi S, Iazzolino E, Barale R 2000. Are baseline frequencies of SCEs, CAs, and MN in human lymphocytes related to hematological values? *Mutat Res.* 469: 159-166.
19. Leopardi P, Zijno A, Marcon F, Conti L, Carere A, Verdina A, Galati R, Tomei F, Baccolo TP, Crebelli R 2003. Analysis of Micronuclei in Peripheral Blood Lymphocytes of Traffic Wardens: Effects of Exposure, Metabolic Genotypes, and Inhibition of Excision Repair In Vitro by ARA-C. *Environ Mol Mutagen.* 41: 126-130.
20. Look KV, Botsivali M, Zangogianni M, Anderson D, Baumgartner A, Fthenou E, Chatzi L, Marcos R, Agramunt S, Namork E, Granum B, Knudsen LE., Nielssen JKS, Meltzer HM, Haugen M, Kyrtopoulos SA, Decordier I, Plas G, Roelants M, Merlo,F, Kleinjans J, Kogevinas M, Kirsch Volders M 2014. The effect of dietary estimates calculated using food frequency questionnaires on micronuclei formation in European pregnant women: a NewGeneris study. *Mutagenesis.* 29: 393-400.
21. Maffei F, Zolezzi Moraga JM, Angelini S, Zenesini C, Musti M, Festi D, Cantelli-Forti G, Hrelia P 2014. Micronucleus frequency in human peripheral blood

- lymphocytes as a biomarker for the early detection of colorectal cancer risk.
Mutagenesis. 29: 221-225.
22. Nefic H and Handzic I. 2013. The effect of age, sex, and lifestyle factors on
micronucleus frequency in peripheral blood lymphocytes of the Bosnian population.
753:1-11.
23. Raaschou-Nielsen O, Andersen ZJ, Beelen R, Samoli E, Stafoggia M, Winmayr G,
Hoffmann B, Fischer P, Nieuwenhuijsen MJ, Brunekreef B, et al. 2013. Air pollution
and lung cancer incidence in 17 European cohorts: prospective analyses from the
European Study of Cohorts for Air Pollution Effects (ESCAPE). *Lancet Oncol.* 14:
813-822.
24. Rossner P, Rossnerova J, Spatova M, Beskid O, Uhlirova K, Libalova H, Solansky
I, Topinka J, Sram JR 2013. Analysis of biomarkers in a Czech population exposed to
heavy air pollution. Part II: chromosomal aberrations and oxidative stress.
Mutagenesis. 28: 97-106.
25. Santovito A, Cervella P, Delperio M 2012. Chromosomal aberrations in cultured
human lymphocytes treated with the fungicide Thiram. *Drug Chem Toxicol.* 35: 347-
351.
26. Santovito A, Cervella P, Delperio M 2016. Baseline frequency of chromosomal
aberrations and sister chromatid exchanges in peripheral blood lymphocytes of healthy
individuals living in Turin (North-Western Italy): assessment of the effects of age, sex
and GSTs gene polymorphisms on the levels of genomic damage. *Ann Hum Biol.* 43:
269-278.
27. Santovito A, Gendusa C, Cervella P 2017. Evaluation of baseline frequency of sister
chromatid exchanges in an Italian population according to age, sex, smoking habits,
and gene polymorphisms. *Am J Hum Biol.* 29: e23034.

- 1
2
3 28. Scarpato R, Verola C, Fabiani B, Bianchi V, Saggese G, Federico G 2011. Nuclear
4 damage in peripheral lymphocytes of obese and overweight Italian children as
5 evaluated by the γ -H2AX focus assay and micronucleus test. *FASEB J.* 25: 685-693.
6
7
8
9
10 29. Villarini M, Levorato S, Salvatori T, Ceretti E, Bonetta S, Carducci A, Grassi T,
11 Vannini S, Donato F, Bonetta S, et al. 2018. Buccal micronucleus cytome assay in
12 primary school children: A descriptive analysis of the MAPEC_LIFE multicenter
13 cohort study. *Int J Hyg Environ Health* 221: 883-892.
14
15
16
17
18
19 30. WHO (World Health Organization) Europe. 2006. Air Quality Guidelines. Global
20 Update 2005. Copenhagen, Denmark. ISBN 92 890 2192 6
21
22
23
24 31. WHO (World Health Organization) Europe. 2007. Children's health and the
25 environment in Europe: a baseline assessment. (Eds: D. Dalbokova, M.
26 Krzyzanowski, S. Lloyd), Copenhagen, Denmark. ISBN: 978 92 890 7297 7
27
28
29
30 32. WHO (World Health Organization) Europe. 2013. Review of evidence on health
31 aspects of air pollution-REVIHAAP Project. Copenhagen, Denmark.
32
33
34
35 33. Ye CJ, Regan S, Liu G, Alemara S, Heng HH. 2018. Understanding aneuploidy in
36 cancer through the lens of system inheritance, fuzzy inheritance and emergence of
37 new genome systems. *Mol Cytogenet* 11:31.
38
39
40
41
42 34. Ye CJ, Sharpe Z, Alemara S, Mackenzie S, Liu G, Abdallah B, Horne S, Regan S,
43 Heng HH. 2019. Micronuclei and Genome Chaos: Changing the System Inheritance.
44 *Genes (Basel)* 10(5):366.
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1 - General characteristics of the studied population

Subjects	N (Frequency)	Age (Mean \pm S.D.)	BH (m \pm S.D)	BM (Kg \pm S.D.)	BMI (Kg/m² \pm S.D.)
Total	150	30.63\pm8.98	1.71\pm0.08	67.80\pm10.88	23.13\pm2.91
Sex					
Males	66 (0.44)	32.71 \pm 8.94	1.72 \pm 0.08	69.21 \pm 10.25	23.41 \pm 2.86
Females	84 (0.56)	29.00 \pm 8.72	1.70 \pm 0.09	66.61 \pm 11.28	22.95 \pm 2.92
Age groups					
21-30	99 (0.66)	25.12 \pm 2.74	1.69 \pm 0.08	64.90 \pm 10.30	22.72 \pm 2.90
31-40	28 (0.19)	35.79 \pm 2.54	1.73 \pm 0.08	73.04 \pm 10.60	24.32 \pm 2.93
41-55	23 (0.15)	48.09 \pm 3.97	1.76 \pm 0.07	73.61 \pm 9.14	23.61 \pm 2.43
BMI					
Normal weight	111 (0.74)	29.32 \pm 8.33	1.71 \pm 0.08	64.34 \pm 9.85	21.83 \pm 2.05
Overweight	39 (0.26)	34.39 \pm 9.79	1.70 \pm 0.08	77.46 \pm 7.28	26.85 \pm 1.36

N = number of studied subjects; BH = body height; BM = body mass; BMI = body mass index;
S.D. = Standard Deviation

Table 2 – Frequency of MNi in the studied population according to sex, age and BMI

Groups	N	Cells	MNi (mean± S.D.)	MNC (mean± S.D.)	MNi/BNCs ± S.D. (‰)	MNC/BNCs ± S.D. (‰)	CBPI ± S.D.
Total	150	150,000	1,08	1,08	7.19±2.51	7.17±2.47	1.71±0.08
Sex							
Males	66	66,000	479	478	7.26±2.28	7.24±2.25	1.705±0.07
Females	84	84,000	599	598	7.13±2.68	7.12±2.65	1.64±0.15
Age groups							
21-30	99	99,000	614	614	6.20±1.83 *	6.20±1.83 *	1.64±0.14 ^{a,b}
31-40	28	28,000	220	220	7.86±1.88 **	7.86±1.88 **	1.70±0.06
41-55	23	23,000	244	242	10.61±2.46	10.52±2.37	1.72±0.05
BMI							
Normal weight	111	111,000	735	734	6.62±2.29 ***	6.61±2.25 ***	1.67±0.13
Overweight	39	38,000	343	342	8.80±2.43	8.77±2.40	1.65±0.12

FARE BENE I CALCOLI DELLA RIGA BMI

BMI = Body mass index; BNCs = Binucleated cells; MNi = micronuclei; MNC = cells with 1 or more micronuclei;
CBPI = Cytokinesis-Block Proliferation Index; S.D. = Standard Deviation;

* significant different with respect to other age groups (P <0.001)

** significant different with respect to 41-55 age group (P <0.001)

*** significant different with respect to overweight group (P <0.001)

^a significant different with respect to 31-40 age group (P = 0.008)

^b significant different with respect to 41-55 age group (P <0.001)

Table 3 - Frequency of NPBs and NBUDs in the studied population according to sex, age and BMI

	N	Cells	NPBs	NBUDs	Frequency of BNCs with NPBs \pm S.D. (‰)	Frequency of BNCs with NBUDs \pm S.D. (‰)
Total	150	150,000	248	310	1.65\pm1.54	2.07\pm1.76
Sex						
Males	66	66,000	115	154	1.74 \pm 1.42	2.33 \pm 1.78
Females	84	84,000	133	156	1.58 \pm 1.63	1.86 \pm 1.72
Age groups						
21-30	99	99,000	111	138	1.12 \pm 1.42 *	1.39 \pm 1.43 *
31-40	28	28,000	69	87	2.46 \pm 1.14	3.11 \pm 1.71
41-55	23	23,000	68	85	2.96 \pm 1.22	3.70 \pm 1.40
BMI						
Normal weight	111	111,000	163	211	1.47 \pm 1.57	1.90 \pm 1.77
Overweight	39	39,000	85	99	2.18 \pm 1.32 **	2.54 \pm 1.64 **

BMI = Body Mass Index; NPBs = nucleoplasmic bridges; NBUDs = nuclear buds; S.D. = Standard Deviation;

*P<0.001, Significantly different with respect to the other two age groups

** P<0.001, Significantly different with respect to normal-weight group

Table 4. - Multiple regression analysis of confounding factors on MNi, NPBs and NBUDs values in lymphocytes of the study groups

CF	MNi frequency			NPBs frequency			NBUDs frequency		
	β -co	P-value	95% CI (Lower) - (Upper)	β -co	P-value	95% CI (Lower) - (Upper)	β -co	P-value	95% CI (Lower) - (Upper)
Sex	-0.14	0.08	(-3.14) - (0.17)	0.08	0.27	(-0.18) - (0.65)	-0.01	0.91	(-0.49) - (0.43)
Age	0.70	<0.001	(0.16) - (0.23)	0.60	<0.001	(0.08) - (0.13)	0.64	<0.001	(0.10) - (0.15)
BMI	0.38	<0.001	(1.32)-(3.03)	0.20	0.01	(0.16)-(1.27)	0.16	0.05	(0.00)-(1.28)

BMI = Body Mass Index; β -co = β -coefficient; CF = Confounding Factor; MNi = micronuclei; NBUDs = nuclear buds; NPBs = Nucleoplasmic bridges

Table 5 - List of the mains pollutants, with their concentrations, measured in the year 2017 in the City of Turin. All values represent mean annual values. In bold are reported the values exceeding the limits set by the Italian law.

Data available on: <http://www.cittametropolitana.torino.it/cms/risorse/ambiente/dwd/qualita-aria/relazioni-annuali/relazione2017.pdf>

Accessed 08-02-2019.

Pollutant	Observed Concentration ($\mu\text{g}/\text{m}^3$)*	Limits set by the Italian law ($\mu\text{g}/\text{m}^3$)
SO ₂	7.50	125
C ₆ H ₆	1.13	5
CO	1.60	10
NO ₂	54	40
O ₃	68	120
PM10	42	40
PM2.5	30	25

* Average values from different detection stations.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1 – Examples of bi-nucleated cells with micronuclei (A-B), tri-nucleated cells with micronucleus (C); tetra-nucleated cell with micronuclei (D); bi-nucleated cells with Nuclear Buds (E) and bi-nucleated cells with Nucleoplasmic Bridges (F).

